Cardiovascular pharmacology

Mechanisms of vasorelaxation induced by oleoylethanolamide in the rat small mesenteric artery

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ABSTRACT

The actions of the anandamide-like mono-unsaturated fatty acid oleoylethanolamide (OEA) were first linked to satiety and control of food intake and recently reported to relax resistance vessels. This study characterizes its vasorelaxant mechanisms. Vasorelaxation to OEA were assessed in third order branches of rat superior mesenteric artery using a wire myograph. The roles of the endothelium, KCa channels, perivascular sensory nerves, NO, cannabinoid receptors, and the phospholipase C (PLC)/ inositol trisphosphate (InsP3) and RhoA/ROCK signalling pathways, were assessed. OEA caused concentration- and endothelium-dependent vasorelaxation (pEC50=6.7 ± 0.1, Rmax=$93.1 \pm 2.5\%$). L-NAME greatly reduced the response (residual relaxation of only $24.6 \pm 12.8\%$). Capsaicin and pertussis toxin significantly reduced the vasorelaxation. Precontraction with KCl abolished the response. TRAM-34 had no effect, but both iberiotoxin and apamin+charybdotoxin markedly shifted the OEA concentration–response curve to the right ($\sim$5-fold). O-1918 but not rimonabant attenuated the vasorelaxation. Both the CB_1 receptor antagonist, AM251 and the CB_2 receptor antagonist, AM630, given alone or in combination, reduced the response to OEA. Inhibition of PLC by U73122, ROCK by Y-27632 and antagonism of inositol trisphosphate (InsP3) receptors by 2-APB abolished OEA vasorelaxation. OEA vasorelaxation involves an endothelial site of action but not the known cannabinoid receptors. It involves Ca^{2+} released from InsP3-sensitive endothelial stores by mechanisms involving RhoA kinase and phospholipase C. It is likely that the released Ca^{2+} causes NO generation and opening of mainly large-conductance K_{Ca} channels. This study demonstrates a possible novel endothelial target that might be important in the control of regional blood flow induced by this lipid molecule.

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1. Introduction

Production of endocannabinoids in biological systems is associated with concomitant release of saturated and mono- or di-unsaturated congeners (monoacylglycerols and N-acylethanolamides) that, while they are proposed to be inactive at cannabinoid receptors, nonetheless influence endocannabinoid metabolism, for example, by inhibiting the anandamide membrane transporter or fatty acid amide hydrolase (FAAH; which metabolises anandamide). They may also interact with non-cannabinoid receptors such as the transient receptor potential V_1 (TRPV$_1$) receptor (see Hanus, 2009 for review).

Acylethanolamides are lipid-signalling molecules widely distributed in plants, invertebrates, and mammals (Schmid et al., 1990). They include, besides anandamide, other ethanolamides

such as oleoyl- (OEA), palmitoyl- (PEA), stearoyl-, linoleoyl-, eicosa-pentaenoyl- and docosahexaenoyl-ethanolamide. The most studied are OEA and PEA which are enzymatically released, together with anandamide, from membrane phospholipid precursors when cells are stimulated (see Borrelli and Izzo, 2009 for review).

At first, actions of OEA were linked to satiety and mechanisms controlling food intake. In rodents, administration of OEA prolongs the time between feeding activity and decreases overall food intake (Rodriguez de Fonseca et al., 2001). This anorexic effect has been linked to interaction with receptors like TRPV$_1$ (transient receptor potential vanilloid-1) where short-term feeding was significantly reduced in a control group compared to a TRPV$_1$-null group (Wang et al., 2005).

OEA activates TRPV$_1$ on perivascular sensory nerves and increases Ca^{2+} signals in human cell lines expressing TRPV$_1$ (Movahed et al., 2005). Strikingly, the production of anandamide is accompanied by substantially higher amounts of PEA and OEA (Bisogno et al., 1997; Fegley et al., 2005) and it has been argued that PEA might protect released anandamide from rapid inactivation

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by an "entourage effect" (Ben-Shabat et al., 1998). The entourage effect has been postulated to occur predominantly through $TRPV_1$. In HEK293 cells expressing human $TRPV_1$, OEA robustly potentiated the effect of anandamide on Ca^{2+} influx (Smart et al., 2002). In addition, Ho et al. (2008) demonstrated that OEA enhanced anandamide-induced vasorelaxation mediated through $TRPV_1$ in the rat mesenteric artery. The possible role of OEA in protecting anandamide against degradation is interesting as mammalian tissues, such as brain, jejunum and liver, contain OEA levels which are higher than those of anandamide (Artmann et al., 2008; Richardson et al., 2007). In vascular tissues (e.g., rat mesenteric arteries), its content exceeds that of anandamide by 100 times (Ho et al., 2008).

OEA has been shown recently to cause vasorelaxation of mesenteric artery branches partially by activating $TRPV_1$ and mediating an entourage action on anandamide vasorelaxation. More recently, attention has been turned to a possible role of cyclooxygenase metabolites in OEA actions in the vasculature (Wheal et al., 2010). Despite these studies, the underlying mechanisms of vasorelaxation of OEA and the cellular pathway involved in this effect remains to be elucidated. Therefore, this study was conducted in order to further clarify the actions of OEA in mesenteric resistance arteries and to investigate possible cellular pathways by which it mediates its actions. The roles of the endothelium, nitric oxide, and Ca^{2+}-sensitive K^+ channels (K_{Ca}) as well as of $TRPV_1$ receptors, cannabinoid CB_1 and CB_2 receptors and the putative endothelial cannabinoid receptor sensitive to O-1918 were examined. Furthermore, the possible involvement of the intracellular phospholipase C (PLC)/inositol trisphosphate ($InsP_3$) pathways and of the RhoA-Rho-associated protein kinase (ROCK) were examined.

2. Materials and methods

2.1. Myograph studies

Male Wistar rats (250–400 g; Charles River UK Ltd, Kent) were killed with an overdose of sodium pentobarbital (120 mg/kg i.p.; Sagatal, Rhône Mérieux, Harlow, Essex). All animal care and use was in accordance with the UK Animal (Scientific Procedures) Act 1986. The mesentery was rapidly removed and placed in ice-cold, gassed (95% O_2/5% CO_2), Krebs–Henseleit solution (pH 7.4) of the following composition (mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; D-glucose, 5.5). Unless otherwise stated, the solution also contained indomethacin (10 μM). Small branches of the superior mesenteric artery (307 ± 6.1 μm diameter, 129 vessels) were dissected, cleaned of surrounding tissues and cut into 2 mm-long segments. These were mounted in a Mulvany–Halpern wire myograph (Danish MyoTechnology, Aarhus, Denmark) maintained at 37 °C in gassed (95% O_2/5% CO_2) Krebs–Henseleit solution.

The arteries were allowed to equilibrate under zero tension for 15–20 min and then were normalized to a tension equivalent to that generated at 90% of the vessel diameter at 100 mmHg (White and Hiley, 1997). The resting force of contraction generated following the normalization process was 3.7 ± 0.1 m N (114 vessels) and the diameter under these conditions was 463 ± 8 μm (129 vessels). After normalization, the vessels were left for another 10–15 min before a test for endothelial integrity which was assessed by submaximal contraction with methoxamine (10 μM), followed by relaxation with carbachol (10 μM). Vessels were considered to have functional endothelium when carbachol (10 μM) reduced methoxamine-induced tone by >90%. If endothelium was not required, it was removed by rubbing the intimal surface with a human hair and vessels which relaxed <10% to carbachol were designated as endothelium-denuded. The mean force of contraction

generated by the vessels in response to methoxamine was 13.3 ± 0.5 m N (108 vessels). Tension was recorded on a PowerLab recording system (ADInstruments, Hastings, Sussex) connected to a Macintosh personal computer.

In order to study the vasorelaxant effects of OEA, vessels were precontracted with methoxamine (10 μM) and once a stable level of tone had been achieved, a cumulative concentration–response curve to OEA was constructed. In some experiments where mechanisms related to endothelium-dependent hyperpolarization were assessed, the arterial segments were precontracted with Krebs–Henseleit solution containing 60 mM KCl as described previously (White and Hiley, 1997). In those experiments where intracellular signalling pathways were explored using inhibitors of PLC and p160ROCK, or an antagonist of $InsP_3$ receptors, it was noted that these compounds inhibited methoxamine-induced contraction. Therefore, vessels were precontracted with a sub-maximal concentration of U46619 (3 μM) either alone, as in case with the inhibitor of PLC and the IP_3 receptor antagonist, or in combination with methoxamine (10 μM) when the Rho-ROCK inhibitor was used. The mean force of contraction induced by U46619 was 11.9 ± 1.4 μM (18 vessels).

In order to study the effect of receptor antagonists, channel blockers or enzyme inhibitors, these were incubated in the bathing solution for 30 min before addition of OEA and then were present during the construction of concentration–response curve. In the case of pertussis toxin, incubation was for 2 h.

Experiments were conducted in a paired fashion, with control and test experiments carried out on arteries from the same animal. Each preparation was only exposed to a single compound unless otherwise mentioned.

2.2. Data and statistical analysis

Relaxation responses are expressed as the percentage relaxation of the tone induced by 10 μM methoxamine, 3 μM U46619 or 60 mM KCl. Data are shown as mean $\pm$ S.E.M. and n represents the number of rats. When a defined maximum response was observed, the data were fitted to a logistic equation of the following form:

$$E = (R_{max}. [A]^{nH})/(EC_{50}^{nH} + [A]^{nH})$$

where E is the reduction in tone, $[A]$ the concentration of the agonist, R_{max} the maximal reduction of established tone, n_H the slope function and EC_{50} the agonist concentration giving half the maximal relaxation. Curve-fitting was carried out using Kaleida-Graph (Synergy Software, Reading, PA, U.S.A.). Where a defined maximal relaxation was not clear, and thus it was not possible to determine an EC_{50}, the $EC_{50\%}$ (the concentration of agonist giving 50% relaxation of the induced tone) was obtained from each individual curve. For comparison purposes both values are expressed as mean pEC_{50} or $pEC_{50\%}$ (p denotes the negative logarithm of the respective concentration). In curves where the value of R_{max} was not clear, mean relaxation achieved at the highest concentration used is given.

Concentration–response curves were analyzed using two-way analysis of variance (ANOVA) of the whole dataset using Prism4 for the Macintosh (GraphPad Software, San Diego, CA, U.S.A.). In experiments where a single concentration of OEA was used, statistical comparisons between individual groups were carried out using one-way ANOVA followed by a Bonferroni post-hoc test; $P < 0.05$ was considered as statistically significant.

2.3. Drugs

Methoxamine, carbachol, atropine, L-nitroarginine methyl ester (L-NAME; all from the Sigma Chemical Company, Poole, Dorset),